Partitioning variation in duration of ant feeding bouts can offer insights into the palatability of insects: experiments on African fruit-feeding butterflies

FREERK MOLLEMAN[1,2*], ANTS KAASIK[1], MELISSA R. WHITAKER[3], JAMES R. CAREY[2]

[1] Institute of Ecology and Earth Sciences, University of Tartu, Vanemuise 46, EE-51014 Tartu, Estonia
[2] Department of Entomology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
[3] Department of Evolution and Ecology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

Abstract. Quantification of chemical defence contributes to the study of animal signals, and to understanding trade-offs among defences and life history traits. Some tropical fruit-feeding butterfly species can be expected to have well-developed anti-predator defences because they are long-lived, are host-plant specialists, and/or have contrasting colourations that may be involved in mimicry relationships. Yet, as a group they are often assumed to be palatable, even without supporting data. Palatability is a continuum that embraces within and between prey-species variation, and therefore, both among- and within-species variation must be documented. Palatability of nine species of fruit-feeding butterfly in Uganda was rated using a novel assay. One hundred and twenty-five butterflies were homogenized, their ground tissues suspended in sugar water and these suspensions offered as small droplets to individual ants in Petri dishes. The time ants spent feeding on these droplets was measured. Danaine butterflies were used as unpalatable references, and sugar solution as a palatable reference. Ants tended to eat in significantly shorter bouts from danaines compared to fruit-feeding species, and feeding bouts on pure sugar solution were longest. Within fruit-feeding species, variation in the duration of ants' feeding bouts was very substantial. There was also considerable variation among individual ants, such that large sample sizes would be needed to reliably distinguish palatability of different species of fruit-feeding butterflies. In explorative analyses, at least three fruit-feeding butterfly species that were assumed palatable appeared to be chemically defended. These results suggest that, in contrast to common assumptions, some tropical fruit-feeding butterflies use unpalatability for defence, perhaps contributing to their long life spans in the wild.

Key words: fruit-feeding, tropical, Nymphalidae, mimicry, colour, chemical defence

INTRODUCTION

Quantification of chemical defence is important for understanding the evolution of signals to predators, investments in other types of defences, and life history. Tropical fruit-feeding butterflies generally have long life spans, with many species

* *Corresponding author.* Institute of Ecology and Earth Sciences, University of Tartu, Vanemuise 46, EE-51014 Tartu, Estonia. *freerkmolleman@hotmail.com*

having longevity records that exceed six months (Kelson, 2008; Molleman *et al.*, 2007, F. Molleman unpublished data), therefore they must have effective anti-predator strategies. Chemical defence in long-lived butterflies in the tropics is demonstrated by several pollen-feeding butterfly species in the genus *Heliconius* that have long active life spans similar to fruit-feeding species (Ehrlich & Gilbert, 1973; Engler-Chaouat & Gilbert, 2007; Pasteels & Grégoire, 1983; Turner, 1971). Such association between chemical defence and long life spans in insects was proposed by Pasteels & Grégoire (1983), and butterflies that use more chemically defended host-plants live longer on average (Beck & Fiedler, 2009). However, in this latter multi-species comparison this effect was not statistically significant (Beck & Fiedler, 2009). One potential explanation for this could be that host-plant chemistry is a poor predictor of adult palatability given varying levels of sequestration and *de novo* synthesis, and thus chemical defence would be better measured using the butterflies themselves (Beck & Fiedler, 2009). Furthermore, many fruit-

feeding butterflies are host-plant specialists (but not all, notable exceptions are grass feeding Satyrines and some *Charaxes*; e.g. DeVries, 1987; Larsen, 1991), a trait associated with the sequestration of defensive chemicals (Nishida, 2002). Moreover, chemically defended species often signal their unprofitability to predators with visual signals such as contrasting colour patterns (aposematism) and these signals can then be mimicked by other species that are not necessarily defended. Such contrasting patterns are found in many fruit-feeding butterfly species and mimicry does occur within this guild, but evidence for the nature of it (Batesian versus Müllerian, evasiveness versus palatability-mediated) is scarce. Therefore, it is likely that these colours function, at least in part, to signal unpalatability to predators.

Despite these reasons to suspect chemical defence in fruit-feeding butterflies, in the literature only a few species of fruit-feeding butterfly have been shown to be chemically defended (e. g. *Euphaedra cyparissa*: Larsen, 2007)—they are typically assumed to rely on evasive flight (Larsen, 1992b; van Someren & Jackson, 1959), crypsis, or eye-spots (Brakefield & Reitsma, 1991; Hill & Vaca, 2004; Marini-Filho & Martins, 2010) instead of chemical defence. It should be tested whether fruit-feeding butterflies that can putatively be classified as evasive (e. g. *Charaxes, Euphaedra*) and/or cryptic (e. g. *Kallimoides, Gnophodes*) also employ chemical defence in the form of unpalatability. Finally, insight into within-species variation in palatability is of interest. Various factors have been implicated in such variation, including age, sex, larval host-plant, and genetic differences in defence strategy (Alonso-Mejia & Brower, 1994; Brower *et al.*, 1982; Eggenberger *et al.*, 1992; Eggenberger & Rowellrahier, 1992, 1993; Holloway *et al.*, 1993; Moranz & Brower, 1998; Saporito *et al.*, 2010).

The present study used the duration of feeding bouts of workers of one ant species on butterfly suspensions as a measure of butterfly palatability. Warningly coloured species were hypothesized to be unpalatable, and chemical defence was hypothesized to play a role in an apparent mimicry relationship within the genus *Euphaedra*: *E. medon* Thurau 1903 females and *E. harpalyce* Talbot 1929 (not closely related within the genus). Mimicry in this group has been suspected to be based on signalling of unprofitability based on evasive flight (van Someren & Jackson, 1959), but strong evidence for such mimicry is lacking (Ruxton *et al.*, 2004). The technique used to measure palatability was convenient in a field setting under tropical conditions, provided values for individual butterflies, was free of prejudice based on any visual signal, was (nearly) independent of odour-mediated attractiveness, and subjects could be assayed over an extended period.

MATERIAL AND METHODS

Study site and subjects

This study was conducted at the Makerere University Biological Field Station in Kibale Forest National Park, Western Uganda. The field station borders selectively logged moist evergreen forest at an altitude of around 1500 m (Chapman *et al.*, 2005). Palatability tests were conducted on nine fruit-feeding butterfly species (Lepidoptera: Nymphalidae) illustrated in Fig. 1. Three species of danaines (Lepidoptera, Nymphalidae) were included (*Amauris niavius* (Linnaeus, 1758), *Tirumala petiverana* (Doubleday 1847), *T. formosa* (Godman 1880)) as examples of unpalatable butterflies (Jeffords *et al.*, 1979) including evidence for African species but not the particular species used (Larsen, 1983, 1992a, 2007). The fruit-feeding butterflies included species with brightly coloured and contrasting wing uppersides (*E. eusemoides* Grose-Smith & Kirby 1889, *E. alacris* Hecq 1979), one species with deep blue/ violet females and metallic green males (*E. kakamega* van Someren 1934), and species with cryptic wing patterns (*Gnophodes chelys* Fabricius 1793, *Kallimoides rumia* Westwood 1850), as well as species that are neither particularly cryptic nor clearly warningly coloured (*E. medon, E. harpalyce, Harma theobene* Doubleday 1849, *Charaxes fulvescens* Aurivillius 1891). Larvae of most of these fruit-feeding butterflies are considered cryptic. However, *Euphaedra* caterpillars may be imprecise mimics of stinging slug-caterpillars (Limacodidae), and contrasting colours are found in the gregarious caterpillars of *E. kakamega* (black with light yellow bands: Molleman & Hecq, 2005) and to a lesser extent in those of *E. eusemoides* (green with dark dorsal setae: Molleman, in press). The combination of warning colours and gregariousness clearly indicates unpalatability (Sillen-Tullberg, 1988), and this is then usually transferred to the adult stage as well (Pasteels & Grégoire, 1983). The host-plants from which the studied species were reared in Kibale (Table 1) belong to families from which various unpalatable or toxic chemicals are known (e.g. Claudino *et al.*, 2009; Dongo *et al.*, 2009; Krief *et al.*, 2006; Penders & Delaude, 1994; Webber & Woodrow, 2009), except for the grass feeding *G. chelys*, while the host-plant of *K. rumia* is still unknown.

Experimental methods

Butterflies were either reared from field-collected caterpillars (most fruit-feeding butterfly individuals except *K. rumia*) or were collected from the field as

adults using sweep nets (danaines) or baited traps (all others). They were killed and the legs, wings, and head were removed before weighing. Each specimen was then ground up with three times its weight of boiled rainwater using a mortar and pestle. The resulting suspension was placed in a vial. In experiment I, a second solution was prepared in another vial that contained the same amount of water as was added to the butterfly suspension, and a ten percent sucrose solution was added to both vials so that both had a five percent sugar concentration. One droplet of each solution/suspension (one pair per Petri dish) was then placed two centimetres apart near the centre of a Petri dish (droplets were named 'butterfly' and 'sugar'). In experiment II, the droplet of sugar solution was omitted but the butterfly suspension was prepared in the same way.

Three ant species that were common in the vicinity were tested in preliminary trials, but only *Myrmicaria* c.f. *natalensis* Smith 1858 (subfamily Myrmicinae) workers walked around quietly and fed from the solutions offered, while the others tended to sit at the edge of the petri-dish without moving or ran about frantically. *M. natalensis* (Smith) (Hym: Formicidae) is a large, slow-moving, predaceous ant that forms large nests of up to several thousand workers (Arnold, 1924) and is very common around Kibale National Park. Worker ants from this species were collected from nearby ant trails and one ant was introduced into each Petri dish with a soft forceps. They were left in the Petri dish for up to 90 minutes, and were replaced after 15 minutes of inactivity. Even though ants may show different behavior in their natural context than when isolated in a Petri dish, workers of this species appeared to be reasonably at ease in our set up. Similar arena trials for example in bio-activity tests of larval sawfly haemolymph of (Müller *et al.*, 2002) and for ranking the strength of interactions within and between species in the context of butterfly-ant mutualism (e.g. Ballmer & Pratt, 1991; Burghardt & Fiedler, 1996) have proven to be highly useful.

Observations were made by teams of two to five local technicians. Each person could simultaneously observe up to six Petri dishes, each containing one pair of droplets (or one droplet in experiment II) and one ant, while one person recorded the data. Start and end times for each ant feeding bout were noted in seconds. Local weather data were used to control for any temperature effect. Experiment I was first supervised by FM (May-June 2007), was then carried on without supervision, and was later supervised by MRW (July-August 2008). Experiment II was performed by local technicians without supervision (September 2008-July 2009).

Table 1. Host-plant information for the assayed butterflies in Kibale National Park, Uganda.

Butterfly species	Host-plant genus	family
Danainae	?	Asclepiadaceae, Euphorbiaceae
Euphaedra kakamega	*Aphania*	Sapindaceae
Euphaedra alacris	*Aphania*	Sapindaceae
Euphaedra eusemoides	*Uvariopsis*	Annonaceae
Euphaedra harpalyce	*Blighia, Aphania, Pancovia*	Sapindaceae
Euphaedra medon	*Paullinia*	Sapindaceae
Harma theobene	*Lindackeria*	Achariaceae
Charaxes fulvescens	*Allophylus*	Sapindaceae
Kallimoides rumia	?	
Gnophodes chelys	*Setaria*	Poaceae

Data analysis

Each ant's first choice of droplet (butterfly vs sugar) in experiment I was recorded. Ants typically returned to the same droplet but some switches were observed as well, and these were expressed as proportions. Statistical analyses were performed using linear mixed models on ant feeding bout durations in R (package lme4: Bates *et al.*, 2011), residuals showing an adequate fit of the modelling approach. Variation among individual ants and variation among butterfly individuals of the same species were captured with random effects, with ants nested in butterfly individual. We attempted to correct for possible differences among experiment days by using weather data as covariates for all data, and we used the duration of feeding bouts on sugar as daily references (for experiment I only). To determine whether a pooled analysis of data from experiments I and II was appropriate (the only difference between them being the presence/absence of the sugar droplet), we compared distributions of durations of two butterfly species that were well represented in both data sets, and compared species effect estimates between separate analyses of experiments I and II.

First, we tested whether fruit-feeding butterflies as a group could be distinguished from the references (danaines and sugar solution) using one-tailed tests. Second, we tested whether there were significant differences among the fruit-feeding butterflies using a two-tailed test. Lastly, effect estimates for butterfly species were calculated and compared to the references using one-tailed tests. To compare
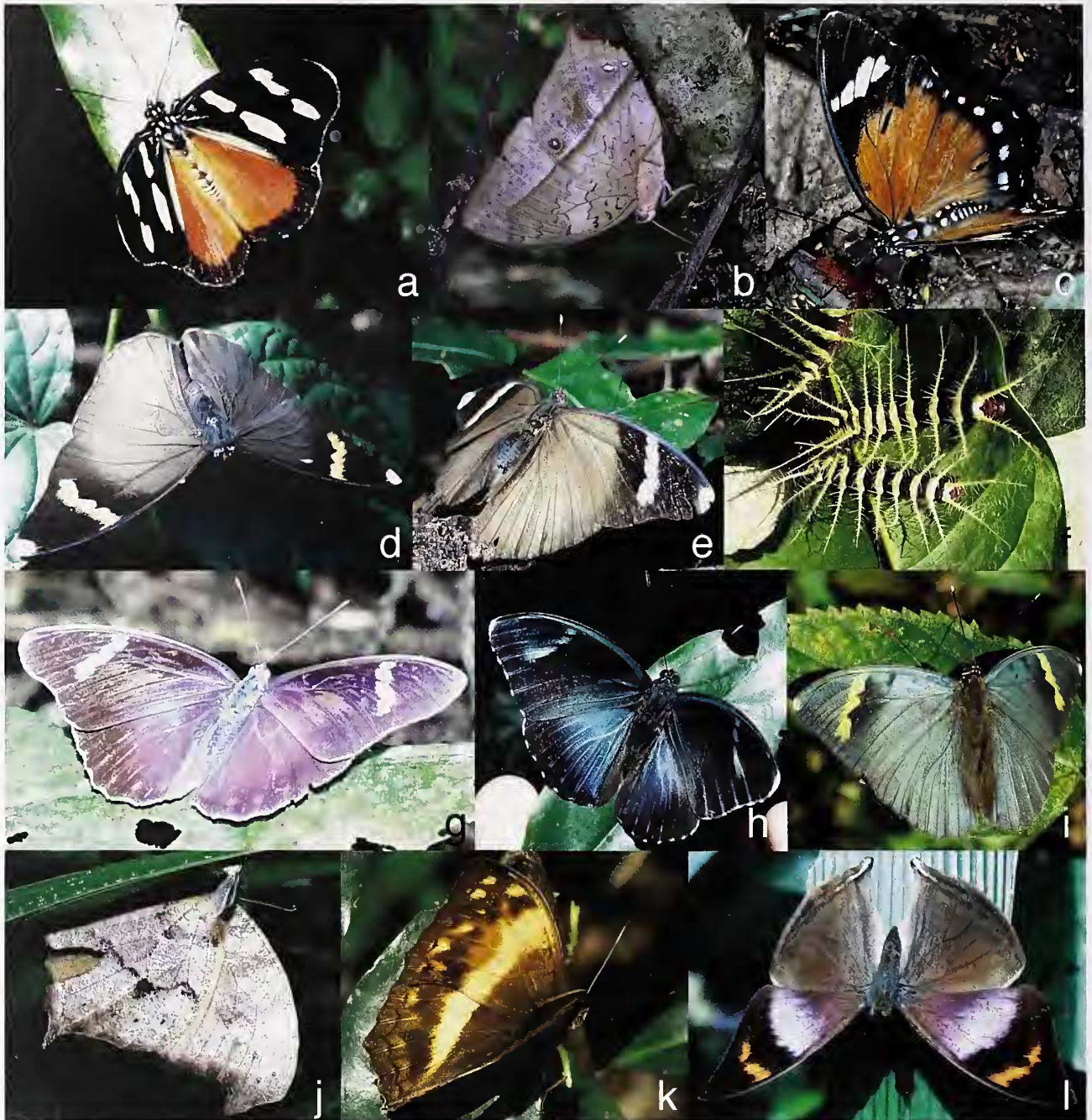
Figure 1: Photographs of butterfly species on which palatability tests were conducted. **a**. *Euphaedra eusemoides* female. **b**. *Charaxes fulvescens* male. **c**. *E. alacris* female. **d**. *E. harpalyce* female. **e**. *E. medon* female. **f**. *E. kakamega* caterpillars. **g**. *E. kakamega* female. **h**. *E. kakamega* male. **i**. *E. medon* male. **j**. *Gnophodes chelys* female. **k**. *Harma theobene* male. **l**. *Kallimoides rumia* male.

durations of feeding bouts on butterfly suspension with those on sugar solution while using durations on sugar to correct for day effects, the feeding bout durations on sugar were subtracted from those on butterfly, and were then tested for equality with zero (data from experiment I only).

RESULTS

Experiment I yielded data from 57 butterflies (9 species), and experiment II from 68 butterflies (8 species) and the pooled data involved 663 feeding ants. The first choice of ants was biased towards

butterfly suspension for all species, including danaines. Switching occurred mostly from sugar to butterfly, except for danaines, *E. kakamega*, and *H. theobene* (Table 2).

Feeding bout durations were seemingly gamma distributed, ranging from a few seconds to about 10 minutes. Log transformation produced a normally distributed response variable that was used for all subsequent analyses and the graphical representation. Modelling log-transformed data using the normal distribution corresponds to the (untransformed) data being from the log-normal distribution. The interval for the mean feeding bout duration within a species could, therefore, be estimated while taking into account the residual variation in the model. For species that were well represented in both datasets, the distribution of durations of feeding bouts on butterfly droplets were very similar (Kolmogorov-Smirnov D=0.11, p=0.42 for *E. alacris* and D=0.10, p=0.24 for *C. fulvescens*). Moreover, the estimates for species effects were similar among the two experiments (Table 3). Therefore, it was justified to combine the data sets for this response variable.

Within-species variation was extensive (Fig. 2). Because the random effects are assumed to be normally distributed, we can analyze this variation in detail. For example, in the analysis of pooled data (Table 3), the standard deviation for the butterfly random effect was 0.54, meaning that for each species, individuals have a high probability of an average that

is up to 1.06 higher or lower than the average for the species, which is a lot because species estimates range between 3.23 and 4.35. The magnitude of the within- species variation can be illustrated further using *E. harpalyce* where the interval that includes about 95% of the individuals is 62 to 520 seconds. It was problematic to include butterfly age (freshly emerged vs field collected) into the model, because of unequal distribution among species and small sample sizes. Graphical representation did not suggest any correlation between age (freshly eclosed vs field collected) and palatability. For the species with the largest sample size (*C. fulvescens*) no significant age effect was detected either. Moreover, no sex effect was found in our data, and including our weather data did not improve our models. The variation among individual ants was also substantial with an SD for the pooled data of 0.37 (Table 3). No effect of order number of feeding of individual ants was detected.

The analysis of feeding bout durations on pure sugar showed that feeding bout durations differed among days, and a similar pattern was detected within the butterfly species such that there was a correlation between feeding bout durations on sugar and butterfly on particular days. However, daily maximum temperature was not correlated with ant feeding bout duration on sugar. Sugar solution was only used in experiment 1, and for these data correcting for such day effects by including durations on sugar as a co-factor improved the performance of

Figure 2. Palatability of butterfly species measured as duration of individual feeding bouts of ants (feedings) on butterfly suspensions (number of freshly emerged and field collected butterflies used). Box-plots represent averages among individual butterflies that are in turn based on varying numbers of feeding bouts with median, quartiles, and full range. The vertical dotted line represents the average duration of feeding bouts on sugar solution.
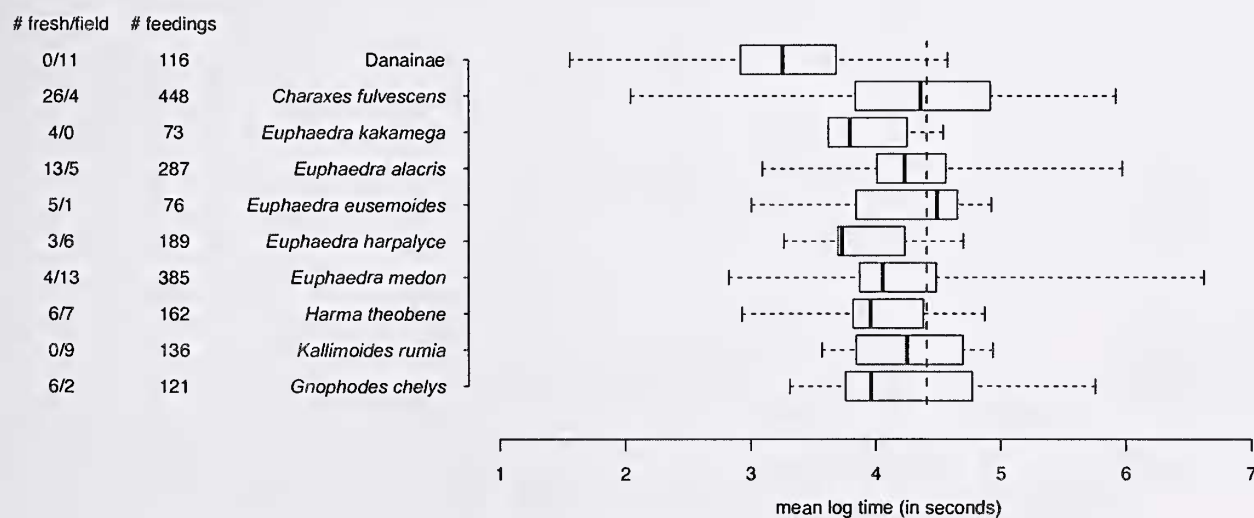
Table 2. Comparisons of ant feeding behaviour on sugar solution and butterfly suspension. For experiment I, choice behaviour is expressed as proportion of ants feeding first on butterfly or sugar solution, and subsequent number and proportion of switches. For feeding bout duration, parameter estimates for the linear mixed model of the log-duration of feeding bouts on butterfly suspension are reported first for experiment I, where for each observation day feeding bout durations on sugar were used as a reference, and second for the pooled data. P-values represent the equality with sugar in one-tailed tests without correction for multiple testing. As a group, fruit-feeding butterflies can be distinguished from sugar (random effects model, p=0.021). This table illustrates that 1) ants are more attracted to butterfly suspension than to pure sugar solution, and those ants starting with butterfly suspension are less likely to switch to sugar than vice versa, except in the case of three butterfly species that were also fed on for short feeding bouts; and 2) some butterfly species are fed on for similar feeding bout durations as sugar solution, while others appear less palatable to ants.

	Experiment I								**Pooled data**			
	1ˢᵗ choice		Switch to			Feeding bout duration corrected for sugar			Distinguishing butterflies from sugar			
N ants	Butterfly	switching	butterfly	sugar	N bouts	estimate	SE	p	N bouts	estimate	SE	*p*
Danainae	13	0.69	2	0.00	0.22	27	-1.21	0.50	0.025	116	-1.17	0.22	<0.001
Euphaedra kakamega	30	0.6	7	0.17	0.28	73	-0.05	0.35	0.878	73	-0.51	0.34	0.073
Euphaedra alacris	49	0.84	10	0.25	0.20	114	0.07	0.24	0.774	287	-0.05	0.16	0.373
Euphaedra eusemoides	43	0.67	8	0.43	0.07	76	0.08	0.29	0.790	76	-0.09	0.28	0.376
Euphaedra harpalyce	18	0.83	4	0.67	0.13	38	-0.95	0.40	0.028	189	-0.55	0.21	0.009
Euphaedra medon	14	0.79	4	0.67	0.18	30	0.77	0.48	0.128	385	-0.29	0.16	0.038
Harma theobene	9	0.56	1	0.00	0.20	12	-0.49	0.58	0.409	162	-0.34	0.19	0.046
Charaxes fulvescens	110	0.79	27	0.43	0.20	262	-0.3	0.16	0.083	448	-0.08	0.12	0.264
Kallimoides rumia	31	0.81	6	0.50	0.12	66	-0.29	0.31	0.358	136	-0.10	0.23	0.338
Gnophodes chelys	25	0.72	5	0.57	0.06	38	-0.35	0.36	0.346	121	-0.17	0.24	0.241
SD (butterfly)						0.56				0.54		
SD (ants nested in butterfly)						0.53				0.37		
SD (residual)						1.18				1.27		

Table 3. Parameter estimates for linear mixed models of the log-duration of feeding bouts on butterfly suspension. P-values show the equality with danaines in one-tailed tests without correction for multiple testing. As a group, fruit-feeding butterflies can be distinguished from danaines (random effects model, p<0.001), and within this group species do not differ significantly from each other (random effects model excluding danaine and sugar data, p=0.54). N = number of feeding bouts, numbers of individual butterflies are given in Figure 2. N* is the estimated number of feeding bouts that needs to measured to distinguish the butterfly species from danaines. This table illustrates that 1) results do not differ much between the two experiments so that pooling of data is justified, 2) random effects are large compared to species effect estimates, showing that there is important variation within species and among ants, 3) all of the fruit-feeding butterflies were fed on for longer bouts than the unpalatable danaines, *E. kakamega* being the only species that was not distinguished, which is attributable to an estimate near danaines as well as low sample size.

	Experiment 1				Experiment 2				Pooled data				
	N	Estimate	SE	*p*	N	Estimate	SE	p	N	Estimate	SE	*p*	N*
Danainae	27	2.29	0.54		89	3.53	0.21		116	3.23	0.22		
Euphaedra kakamega	73	3.91	0.39	0.012					73	3.89	0.34	**0.058**	81
Euphaedra alacris	114	4.48	0.26	0.001	173	4.24	0.17	0.008	287	4.35	0.16	<0.001	52
Euphaedra eusemoides	76	4.33	0.31	0.002					76	4.31	0.28	0.003	25
Euphaedra harpalyce	38	4.23	0.44	0.006	151	3.68	0.20	**0.298**	189	3.85	0.21	0.030	142
Euphaedra medon	30	4.36	0.53	0.007	355	4.06	0.13	0.020	385	4.11	0.16	0.002	111
Harma theobene	12	3.11	0.62	**0.167**	150	4.17	0.16	0.013	162	4.06	0.19	0.005	61
Charaxes fulvescens	262	4.51	0.18	<0.001	186	4.09	0.16	0.023	448	4.32	0.12	<0.001	73
Kallimoides rumia	66	4.53	0.33	0.001	70	3.94	0.30	**0.138**	136	4.30	0.23	0.002	37
Gnophodes chelys	38	4.61	0.39	0.001	83	3.92	0.25	0.120	121	4.23	0.24	0.003	39
SD(butterfly)		0.60				0.38				0.54			
SD(ants nested in butterfly)		0.46				0.29				0.37			
SD(residual)		1.21				1.31				1.27			

the statistical test. Nevertheless, pooled analyses for comparing fruit-feeding butterflies to danaines and to sugar solution remained preferred because of the larger sample size.

Ant feeding bouts were shortest for danaine specimens on average (Fig. 2), and as a group, fruit-feeding butterflies could be distinguished from them (random effects model, $p<0.001$). On average, sugar was fed on for longer bouts than the fruit-feeding butterflies combined (random effects model, $p=0.021$). Some fruit-feeding butterfly species were fed on for shorter bouts than others on average (Fig. 2), but these differences were not significant (random effects model excluding danaine and sugar data, $p=0.54$). In exploratory analyses (without correcting for multiple testing) *E. harpalyce*, *E. medon*, and *H. theobene* could be distinguished from sugar (Table 2), and *E. kakamega* could not be distinguished from danaines (Table 3). However, non-significance is attributable to low sample sizes, and estimates of the number of feeding bouts that need to be measured to statistically distinguish the species from the assayed danaines (N*) may be best suited to preliminarily rank the species according to palatability. This suggests that *E. harpalyce* and *E. medon* are least palatable, followed by *E. kakamega* (Table 3).

DISCUSSION

We showed that certain African fruit-feeding butterflies can be moderately unpalatable to ants, and documented extensive variation in palatability within butterfly species. To interpret colour patterns and discover trade-offs with other defences and life history traits, it would be useful to know the palatability of butterflies to the relevant predators. However, observations of predation on fruit-feeding butterflies in the wild are extremely rare. If natural predators are visual hunting vertebrates (e.g. birds and lizards) as can be suspected, it can be hard to obtain palatability values for individual prey items that are unbiased by prey appearance and predator experience. Hence, assays with insects such as ants are useful (e. g. Eisner *et al.*, 2008). Given the diversity of potential predators in tropical forests, we expect that tropical butterflies that depend on unpalatability for survival should be distasteful to a wide range of predators, including ants. Moreover, congruence among predator species in their responses to defensive chemicals is usually substantial (Pasteels & Grégoire, 1983). For example, hornets, cats and humans ranked the taste of bird meat similarly (Cott, 1947). However, rarely is palatability of butterflies rated using more than one potential predator (Trigo, 2000), and differences in

type of chemical defence most effective to different classes of predators have been noted as well. For example, *Paederus* beetle larvae produce pederin as a defense against spiders, which is not effective against other arthropods (Kellner & Dettner, 1996). While realizing the need for further tests, we interpret shorter feeding bouts of ants as indicative of lower palatability to most generalist predators.

The first droplet that ants fed on was usually butterfly suspension, which may represent a preference or simply an effect of detectability as the butterfly suspension may emit a stronger odour. Therefore, cafeteria experiments where ants choose between food sources do not measure only palatability, but also detectability. Ants' switching behaviour was consistent with the results of feeding bout durations: butterfly species that were fed on for short feeding bouts were also more often switched away from.

The duration of ant feeding bouts varied considerably for individual butterflies of the same species and for individual ants. However, in our data we did not find within-species correlations between ant feeding bout durations and butterfly age or sex. Apart from variation in defensive chemistry, variation in nutritional value (depending on reproductive history and nutritional status of the individual butterfly) could also have contributed to this within-species variation in palatability albeit mainly in the minority of butterflies that had been field-captured. Ant behaviour could vary according to local climate, lineage-specific traits, as well as nutritional status of the colony or individual (e. g. whether an individual was going out to forage or was coming back with food). However, the nutritional status of individual ants did not appear to affect feeding bout duration because the order number of feeding of individual ants did not affect it.

When feeding bout durations on sugar droplets could be used as reference for observation days, some of this variation could be accounted for, leading to greater power for distinguishing butterfly species. This suggests that day-to-day variation in our data is mainly caused by weather, despite lack of correlation with the daily maximum temperature.

Based on the duration of ant feeding bouts, fruit-feeding butterflies are on average more palatable than danaines. As a group fruit-feeding butterflies could be distinguished from the danaines, and all except *E. kakamega* were distinguishable in the exploratory analysis. The interpretation of tests against the pure sugar solution is less straightforward because the sugar solution offers only sugar and water, while the butterfly suspension offers the ants the same sugar concentration, but with added nutrients as well as

defensive chemicals from the butterfly. Thus, the value of a sugar control may lay primarily in its role in accounting for variation in ant behaviour. While, as a group, fruit-feeding butterflies were fed on for significantly shorter bouts than pure sugar solution, differences for several species were small and only three fruit-feeding species (*E. harpalyce*, *E. medon*, and *H. theobene*) were distinguished in the exploratory analysis.

Results of the exploratory analyses are biologically interpretable. The similarity between *E. medon* females and *E. harpalyce* may be an example of Müllerian mimicry, because both species were less palatable than sugar solution and were the hardest to distinguish from Danaines (N* 142 and 111 feedings, respectively). *E. kakamega* also appeared to be unpalatable, and this is not surprising because of its warningly coloured gregarious caterpillars. The adult dorsal wing colouration is also non-cryptic and may be mimicked by other species, most notably *E. uganda* (Aurivillius 1895). *H. theobene* was also distinguished from sugar in the exploratory analyses but appeared more easily distinguished from danaines. Any chemical defence in this species may be related to the suspected sequestration of host-plant chemicals (probably cyanides) in this group (van Velzen *et al.*, 2007), but is surprising in the light of its rather cryptic appearance. This may demonstrate that chemical defence is not always advertised with contrasting colour patterns (Endler & Mappes, 2004). We have noted adverse reactions to other cryptic fruit-feeding butterflies (Molleman *et al.* 2010) but this did not bear out in our analyses of feeding bout durations. On the other hand, contrastingly coloured species *E. alacris* and *E. eusemoides* did not appear to be particularly unpalatable, and these are more likely Batesian mimics of chemically defended moths. However, all such hypotheses on particular species generated by our exploratory analyses need to be tested with further palatability assays, and, most critically, observations on avoidance behaviour of potential predators such as birds and chameleons. Nevertheless, our results indicate that one cannot assume that fruit-feeding butterflies are all equally palatable, despite strongly developed evasive flight and crypsis in this group.

Obtaining palatability data needed to elucidate the evolution of defence and signals such as colour patterns, and their relationship to life history evolution is challenging. It is important to distinguish between tests that measure (innate or learned) responses to appearance, odour, palatability, toxicity, or a combination of these. Moreover, variation within species may also be extensive and of biological interest, and therefore, assays that produce palatability estimates for individuals are preferred over those that yield only a population mean with confidence interval. Given that there is a gradient rather than a dichotomy of palatability (Brower *et al.*, 1968), continuous measures such as feeding bout durations more readily provide statistically significant results. Using common omnivorous and easy-to-handle ants, we presented such a method, that is convenient in a field-lab setting (also in tropical regions) and requires little training.

However, we suggest several improvements to the method presented. Responses of animals used in assays vary over time and among individuals and lineages, and this can to be countered by; 1) using positive and negative controls with each test, preferably for each ant; 2) using worker ants from several documented colonies; 3) only picking individuals that leave the colony on a foraging trail; and 4) recording local conditions (e.g. temperature and humidity for each assay). While a sugar solution is a straightforward palatable control, a more standard negative control would be preferable. If a freezer is available, a stock suspension made from a large number of known unpalatable insects could serve as such. However, to compare palatability among different regions, a global standard of 'mixed defensive chemicals', needs to be developed. Animals behave differently towards potential food in a natural setting than when isolated in a Petri dish. With video cameras ant feeding bouts could be recorded in cafetaria experiments in a natural setting. It can be expected that a much wider range of ant species would be amendable for such approach. This is important when attempting to measure feeding bout durations where such docile ants are not available, and also to test for congruence in the responses of multiple ant species. Moreover, with video images it would be easier to code transient behaviours such as running away from food and grooming, and to distinguish between active feeding and resting at the food. While it remains to be shown that other ant species also adapt their feeding bout durations according to palatability of the food, we believe that this parameter is a relatively efficient metric for ranking the palatability of animals.

ACKNOWLEDGEMENTS

LITERATURE CITED

ALONSO-MEJIA, A. & BROWER, L.P. 1994. From model to mimic: age-dependent unpalatability in monarch butterflies. Experientia, 50, 176-181.

ARNOLD, G. 1924. A monograph of the Formicidae of South Africa. Annals of the South African Museum, 14, 1-766

BALLMER, G.R. & PRATT, G.F. 1991. Quantification of ant attendance (myrmecophily) of Lycaenid larvae. Journal of Research on the Lepidoptera, 30, 95-112.

BATES, D., MAECHLER, M., & BOLKER, B. 2011. lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-42. http://CRAN.R-project.org/package=lme4.

BECK, J. & FIEDLER, K. 2009. Adult life spans of butterflies (Lepidoptera: Papilionoidea plus Hesperioidea): broadscale contingencies with adult and larval traits in multi-species comparisons. Biological Journal of the Linnean Society, 96, 166-184.

BRAKEFIELD, P.M. & REITSMA, N. 1991. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. Ecological Entomology, 16, 291-303.

BROWER, L.P., RYERSON, W.N., COPPINGER, L., & GLAZIER, S.C. (1968) Ecological chemistry and palatability spectrum. Science, 161, 1349-&.

BROWER, L.P., SEIBER, J.N., NELSON, C.J., LYNCH, S.P., & TUSKES, P.M. 1982. Plant-determined variation in the cardenolide content, thin-layer chromatography profiles, and emetic potency of monarch butterflies, *Danaus plexippus* Lepidoptera, Danaidae reared on the milkweed, *Asclepias eriocarpa* Apocynales, Asclepiadaceae in California. Journal of Chemical Ecology, 8, 579-633.

BURGHARDT, F. & FIEDLER, K. 1996. The influence of diet on growth and secretion behaviour of myrmecophilous *Polyommatus icarus* caterpillars (Lepidoptera: Lycaenidae). Ecological Entomology, 21, 1-8.

CHAPMAN, C.A., CHAPMAN, L.J., STRUHSAKER, T.T., ZANNE, A.E., CLARK, C.J., & POULSEN, J.R. 2005. A long-term evaluation of fruiting phenology: importance of climate change. Journal of Tropical Ecology, 21, 31-45.

COTT, H.B. 1947. The edibility of birds: illustrated by 5 years experiments and observations (1941-1946) on the food preferences of the hornet, cat and man - and considered with special reference to the theories of adaptive coloration. Proceedings of the Zoological Society of London, 116, 371-379.

DEVRIES, P.J. 1987. The butterflies of Costa Rica and their natural history. Papilionidae, Pieridae and Nymphalidae Princeton University Press, New Jersey.

DONGO, E., HUSSAIN, H., MIEMANANG, R.S., TAZOO, D., SCHULZ, B., & KROHN, K. 2009. Chemical constituents of *Klainedoxa gabonenses* and *Paullinia pinnata*. Records of Natural Products, 3, 165-169.

EGGENBERGER, F., DALOZE, D., PASTEELS, J.M., & ROWELLRAHIER, M. 1992. Identification and seasonal quantification of defensive secretion components of *Oreina gloriosa* (Coleoptera, Chrysomelidae). Experientia, 48, 1173-1179.

EGGENBERGER, F. & ROWELLRAHIER, M. 1992. Genetic component of variation in chemical defense of *Oreina gloriosa* (Coleoptera, Chrysomelidae). Journal of Chemical Ecology, 18, 1375-1404.

EGGENBERGER, F. & ROWELLRAHIER, M. 1993. Physiological sources of variation in chemical defense of *Oreina gloriosa* (Coleoptera, Chrysomelidae). Journal of Chemical Ecology, 19, 395-410.

EHRLICH, P.R. & GILBERT, L.E. 1973. Population structure and dynamics of the tropical butterfly *Helicoinius ethilla*. Biotropica, 5, 69-82.

EISNER, T., SCHROEDER, F.C., SNYDER, N., GRANT, J.B., ANESHANSLEY, D.J., UTTERBACK, D., MEINWALD, J., & EISNER, M. 2008. Defensive chemistry of lycid beetles and of mimetic cerambycid beetles that feed on them. Chemoecology, 18, 109-119.

ENDLER, J.A. & MAPPES, J. 2004. Predator mixes and the conspicuousness of aposematic signals. American Naturalist, 163, 532-547.

ENGLER-CHAOUAT, H.S. & GILBERT, L.E. 2007. De novo synthesis vs. sequestration: negatively correlated metabolic traits and the evolution of host plant specialization in cyanogenic butterflies. Journal of Chemical Ecology, 33, 25-42.

HILL, R.I. & VACA, J.F. 2004. Differential wing strength in *Pierella* butterflies (Nymphalidae, Satyrinae) supports the deflection hypothesis. Biotropica, 36, 362-370.

HOLLOWAY, G.J., DEJONG, P.W., & OTTENHEIM, M. 1993. The genetics and cost of chemical defense in the 2-spot ladybird (*Adalia bipunctata* L). Evolution, 47, 1229-1239.

JEFFORDS, M.R., STERNBURG, J.G., & WALDBAUER, G.P. 1979. Batesian Mimicry: field demonstration of the survival value of pipevine swallowtail and monarch color patterns. Evolution, 33, 275-286.

KELLNER, R.L.L. & DETTNER, K. 1996. Differential efficacy of toxic pederin in deterring potential arthropod predators of *Paederus* (Coleoptera: Staphylinidae) offspring. Oecologia, 107, 293-300.

KELSON, R. 2008. Searching for Methuselah: butterfly longevity revisited. Proceedings of the invertebartes in education and conservation conference, 51-57.

KRIEF, S., HUFFMAN, M.A., SEVENET, T., HLADIK, C.M., GRELLIER, P., LOISEAU, P.M., & WRANGHAM, R.W. 2006. Bioactive properties of plant species ingested by chimpanzees (*Pan troglodytes schweinfurthii*) in the Kibale National Park, Uganda. American Journal of Primatology, 68, 51-71.

LARSEN, T.B. 1983. On the palatability of butterflies. Entomologist's records, 95, 66-67.

LARSEN, T.B. 1991. The butterflies of Kenya and their natural history Oxford University Press, Oxford.

LARSEN, T.B. 1992a. A chameleon as predator of butterflies and its avoidance of known aposematic species. Tropical Lepidoptera, 3, 101-104.

LARSEN, T.B. 1992b. The Forktailed Drongo *Dicrurus adsimilis* chasing a large butterfly. Babbler, 23, 46-48.

LARSEN, T.B. 2007. Aposematism, mimicry, chameleons and butterflies: a challenging research opportunity. Metamorphosis, 17, 99-107.

MARINI-FILHO, O. & MARTINS, R.R. 2010. Nymphalid butterfly dispersal among forest fragments at Serra da Canastra National Park, Brazil. Journal of Insect Conservation, 14, 401-411.

MOLLEMAN, F. (IN PRESS) Butterflies of Kibale Forest: a picture guide to Ugandan forest butterflies Tourguide publications. An imprint of Fountain Publishers, Kampala.

MOLLEMAN, F. & HECQ, J. 2005. Host plant records and photographs of immature *Euphaedra* from Kibale National Park, Uganda. Lambillionea, CV, 423-429.

MOLLEMAN, F., ZWAAN, B.J., BRAKEFIELD, P.M., & CAREY, J.R. 2007. Extraordinary long life spans in fruit-feeding butterflies can provide window on evolution of life span and aging. Experimental Gerontology, 42, 472-482.

MORANZ, R. & BROWER, L.P. 1998. Geographic and temporal variation of cardenolide-based chemical defenses of queen butterfly (*Danaus gilippus*) in northern Florida. Journal of Chemical Ecology, 24, 905-932.

MÜLLER, C., BOEVÉ, J.L., & BRAKEFIELD, P. 2002. Host plant derived feeding deterrence towards ants in the turnip sawfly *Athalia rosae*. Entomologia Experimentalis Et Applicata, 104, 153-157.

NISHIDA, R. 2002. Sequestration of defensive substances from plants by Lepidoptera. Annual Review of Entomology, 47, 57-92.

PASTEELS, J.M. & GRÉGOIRE, J.C. 1983. The chemical ecology of defense in arthropods. Annual Review of Entomology, 28, 263-289.

PENDERS, A. & DELAUDE, C. 1994. Structure elucidation of an acetylated saponin of *Blighia welwitschii* by nmr-spectroscopy. Carbohydrate Research, 263, 79-88.

RUXTON, G.D., SPEED, M., & SHERRATT, T.N. 2004. Evasive mimicry: when (if ever) could mimicry based on difficulty of capture evolve? Proceedings of the Royal Society of London Series B-Biological Sciences, 271, 2135-2142.

SAPORITO, R.A., DONNELLY, M.A., MADDEN, A.A., GARRAFFO, H.M., & SPANDE, T.F. 2010. Sex-related differences in alkaloid chemical defenses of the dendrobatid frog *Oophaga pumilio* from Cayo Nancy, Bocas del Toro, Panama. Journal of Natural Products, 73, 317-321.

SILLEN-TULLBERG, B. 1988. Evolution of gregariousness in aposematic butterfly larvae: a phylogenetic analysis. Evolution, 42, 293-305.

TRIGO, J.R. 2000. The chemistry of antipredator defense by secondary compounds in neotropical Lepidoptera: Facts, perspectives and caveats. Journal of the Brazilian Chemical Society, 11, 551-561.

TURNER, J.R.G. 1971. Experiments on the demography of tropical butterflies 11: Longevity and homerange behaviour in *Heliconius erato*. Biotropica, 3, 21-31.

VAN SOMEREN, V.G.L. & JACKSON, T.H.E. 1959. Some comments on protective resemblance amongst African Lepidoptera. Journal of the Lepidopterists' Society, 13, 121-150.

VAN VELZEN, R., BAKKER, F.T., & VAN LOON, J.J.A. 2007. DNA barcoding reveals hidden species diversity in *Cymothoe* (Nymphalidae). Proceedings of the Netherlands Entomological Society meetings 18, 95-103.

WEBBER, B.L. & WOODROW, I.E. 2009. Chemical and physical plant defence across multiple ontogenetic stages in a tropical rain forest understorey tree. Journal of Ecology, 97, 761-771.